Transfusion-Associated Microchimerism in Combat Casualties

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Background: **Fresh whole blood (FrWB) is routinely used in the resuscitation of combat casualties in Operation Iraqi Freedom and Operation Enduring Freedom. However, studies have shown high rates (20%–40%) of transfusion-associated microchimerism (TA-MC) in civilian trauma patients receiving allogenic red blood cell (RBC) transfusions. We explored the incidence of TA-MC in combat casualties receiving FrWB compared with patients receiving standard stored RBC transfusions.**

Methods: **Prospective data on TA-MC at ≥14 days posttransfusion were collected on 26 severely injured combat casualties admitted to the National Naval Medical Center between December 2006 and March 2007. Demographic variables included age, sex, Injury Severity Score, and transfusion history. Data are expressed as mean ± SD.**

Results: **The mean age of the study cohort was 24 ± 7; mean Injury Severity Score was 17 ± 12. All were men and suffered penetrating injury. Average hospital length of stay was 46 ± 35 days. TA-MC was present in 45% (10 of 22) patients who were transfused at least 1 unit of blood. The four nontransfused patients all tested negative for TA-MC. Among six patients who received 4 to 43 units of FrWB, five also received RBCs and one aphaeresis platelets. The remaining 16 transfused patients who received RBCs (no FrWB) included seven who also received platelets in theater. The prevalence of TA-MC was 50% (3 of 6) in FrWB patients, 50% in patients given platelets (4 of 8), and 38% (3 of 8) in those given only RBCs as a cellular component ($p = 0.61$).**

Conclusions: **Although these preliminary data do not demonstrate a significantly increased rate of TA-MC in FrWB or apheresis platelets recipients compared with RBC recipients, the overall 45% (10 of 22) rate of TA-MC in transfused soldiers warrants further study to ascertain possible clinical consequences such as graft-versus-host or autoimmune disease syndromes.**

Key Words: **Microchimerism, Transfusion, Combat, Blood, Allogenic, Autologous, T cell.**

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Multiple studies have documented the immunomodulatory effects of blood transfusions, and these effects are evident in both allogenic and autologous blood transfusions.[1–3] They include decreased T-cell proliferation along with decreases in the number of CD3, CD4, and CD8 T cells. Natural killer cell activity is also reduced. Tumor necrosis factor (TNF)-α and its soluble cytokine receptor are increased. There are increases in suppressor T-cell activity, cell-mediated lympholysis, and serum neopterin as well.[4]

Similar immunologic and immunosuppressive effects have been seen in clinical studies. In patients undergoing surgery for colorectal cancer, both allogenic and autologous blood transfusions produce immune activation. They resulted in an increase in soluble interleukin (IL)-2 receptor and increases in TNF-receptors p55 and p75. In addition, allogenic transfusions resulted in an increase in TNF-α.[5] Furthermore, both allogenic and autologous blood transfusions result in significant increases in serum concentrations of IL-6 and IL-8 in patients undergoing total hip replacements. In these patients, the autologous transfusion group had a significantly greater increase in these cytokines compared with the allogenic transfusion group. Both groups also resulted in a significant increase in C3a.[6]

A potential consequence of this immunomodulation and immunosuppression resulting from blood transfusion is the development of microchimerism (MC).[7] Chimerism is defined as the stable persistence of a population of cells from one individual within another.[8,9] When the chimeric population is small (<5%), the term MC is often used and when it is associated with transfusion it is referred to as transfusion-associated MC (TA-MC). Although high levels of chimerism can be intentionally induced through hematopoietic stem cell and organ transplantation under immunosuppressive conditioning, MC can also occur naturally with pregnancy and twinning.[10,11] The prevalence of MC is increased in transfused patients and increased further in transfused trauma patients compared with naturally occurring MC.[12]

Fresh whole blood (FrWB) transfusion has largely been abandoned by civilian institutions in favor of specific blood

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component therapy.[13] However, the military has consistently used FrWB in combat environments for years.[14,15] In Iraq, from 2004 to 2006, approximately 2.1% of all U.S. military trauma patients (12% of all transfused patients) received at least 1 unit of FrWB and overall 1,576 units of FrWB have been transfused.[12,15] Given the current use of FrWB in theater, we sought to determine the prevalence of TA-MC in combat casualties from Operation Iraqi Freedom and Operation Enduring Freedom receiving FrWB compared with patients receiving standard blood product transfusions.

PATIENTS AND METHODS

Prospective data were collected on 26 severely injured patients admitted to the National Naval Medical Center (NNMC) during a 4-month period from December 2006 to March 2007. Patients were stratified by age, gender, mechanism of injury, and transfusion history. Injury severity was assessed using the Injury Severity Score (ISS). The institutional review board at NNMC approved the study and informed consent was obtained from each participant or their legally designated next of kin. All enrolled patients received a unique identifier that resulted in blinding of the investigators performing the laboratory analysis as to the identity of the patient and clinical history. The identifier allowed for linkage of the transfusion history, clinical history, and laboratory results of the patients for future analysis. The investigators did not know the transfusion histories of these patients until the samples had been analyzed for the presence of MC, further blinding the investigators from any possibility of bias. The Armed Services Blood Program Office supplied complete transfusion histories on all patients.

Fresh Whole Blood

FrWB was only collected and transfused at Level II and Level III facilities in theater. No FrWB was collected or transfused at Level IV or Level V facilities because of the availability of adequate component products. In theater, FrWB was collected and transfused only during mass casualty situations or massive transfusions when component therapy was unavailable. Upon determining the need for FrWB, a walking blood bank was initiated. These donors consisted of individuals who had been regularly screened for infections, tested for human immunodeficiency virus (HIV), and whose vaccinations were up to date. Blood products were tested using rapid assays for hepatitis B and C and HIV-1/2 antibodies. Additional samples were subsequently transported back to the United States for repeat formal testing. Approximately 450 mL of whole blood was drawn and placed in a commercial blood bag containing 63 mL of a standard anticoagulant. The blood was then immediately transfused. The time delay from the initiation of the walking blood bank until transfusion of the first unit of FrWB was approximately 1 hour, and all units greater than 24 hours that had not been transfused were destroyed. A similar process was used for the collection and transfusion of apheresis platelets (aplts).

To determine whether fresh product transfusion (FrWB or aplts) was a factor in developing TA-MC, the patients were divided into various groups based on type of blood product transfusion they received. The FrWB group consisted of individuals who received FrWB, pooled plts, and red blood cells (RBCs). The aPLT group consisted of patients who received aplts, pooled plts, and RBCs. The RBC group received only red cell transfusions. The rate of TA-MC was then determined in each group and compared with the rate of TA-MC in the RBC group to determine whether fresh products were a factor in the development of MC.

Specimen Acquisition and Assessment

Enrolled patients underwent evaluation for TA-MC by testing a blood sample obtained at least 14 days after their last transfusion but before discharge from NNMC. The ward staff performed phlebotomy and attempts were made to time the specimen with routine phlebotomies performed for patient care. Each subject had a single 7-mL acid-citrate-dextrose (ACD)-anticoagulated blood specimen drawn at the bedside exclusively for TA-MC analysis to minimize the likelihood of specimen contamination. Whole blood specimens were immediately transported to the NNMC blood bank/transfusion service, where they were mixed and ten 0.5-mL aliquots were dispensed using single-use pipettes into screw-cap cryovials. These aliquots, labeled with a subject code, were immediately frozen at −40°C and subsequently batch-shipped on dry ice to Blood Systems Research Laboratory in San Francisco, CA, where analysis for TA-MC was performed.

Polymerase chain reaction (PCR) analysis for TA-MC was performed at Blood Systems Research Institute by investigators blinded to the identity or clinical status of these subjects. DNA was initially extracted from two aliquots of frozen whole blood. One was used to type the recipients and detect any MC populations using the 12-member human leukocyte antigen (HLA)-DR panel, and the other using the 12-member InDel panel, as described elsewhere.[16,17] In brief, DNA lysates were amplified using sequence-specific primers for a panel of HLA-DR and InDel alleles. The presence of donor leukocytes was determined by comparing the PCR cycle numbers at which DNA corresponding to different DR and InDel alleles were detected by fluorescence of an intercalated dye, SYBR Green. The patient's DR and InDel genotypes were ascertained based on observing amplified product with corresponding primers at a relatively early cycle, whereas nonrecipient DR and InDel alleles present in smaller subpopulations of microchimeric leukocytes, if present, would be detected as amplified products appearing at later cycles. Each of the 12 DR and 12 InDel polymorphisms were amplified in duplicates. Subjects were classified as reactive for MC if any one of the replicates tests had a positive result for a minor population (informative) allele, or negative if all tests showed no evidence of TA-MC. Confirmatory testing was performed on all positive tests by amplifying 12 replicates from additional frozen aliquots for the reactive polymorphism(s)

detected during the screening. One or more additional positive results out of 12 replicates was interpreted as confirmatory for TA-MC. Quantification was performed if six or more reactions were positive, based on running a standard curve, as detailed elsewhere. With a sensitivity of 96% and specificity of virtually 100% using the above confirmatory algorithm, it is unlikely that misclassification of PCR results presented difficulty.

Statistical Analysis

The Fisher's exact test was used to compare the FrWB group and the aPLT group with the standard RBC group to determine differences in the rate of MC. Continuous variables were compared using Student's *t* test. Differences were considered significant when $p < 0.05$. (Stata, Release 6.0; Stata Corp., College Station, TX).

RESULTS

Patient Demographics

The study cohort consisted of 26 patients with a mean age of 24 ± 7 and a mean ISS of 17 ± 12. All were men and sustained penetrating trauma. The mean hospital length of stay was 46 ± 35 days with 85% (n = 22) receiving at least one blood transfusion (Table 1). MC was present in 45% (n = 10) of patients receiving at least 1 unit of blood transfusion. In patients not receiving a blood transfusion, the rate of MC was 0% (n = 4). There were no significant differences in age, gender, or injury severity between MC and non-MC patients. The two groups were also similar in several aspects relating to transfusion, including incidence of transfusion (75% vs. 100%, $p = 0.14$), the number of units transfused (26 ± 26 units vs. 30 ± 53 units, $p = 0.81$), the length of time from injury to blood draw (42 ± 34 days vs. 35 ± 22 days, $p = 0.29$), and the length of time from last transfusion to blood draw (24 ± 11days vs. 21 ± 12 days, $p = 0.27$; Table 2).

Table 1 Patient Demographics (n = 26)

Variable	
Age (yr)	24 ± 7
Injury Severity Score	17 ± 12
Hospital length of stay (d)	46 ± 35
Gender (% male)	100
Penetrating (%)	100
Transfusion (%)	85

Age, Injury Severity Score, and hospital length of stay data presented as mean ± SD.

Transfusion and MC

Transfused blood products included 105 units of FrWB, 54 units of plts (26 units of fresh aplts and 28 units of pooled plts), and 455 units of RBCs (Fig. 1). Patients transfused with FrWB (n = 6) received between 4 and 43 units of FrWB; five of these patients also received RBCs and 1 aplts. The remaining 16 transfused patients who received RBCs (no FrWB) included six who also received plts in theater or in Germany. The FrWB group consisted of patients transfused with FrWB, pooled plts, and RBCs. The aPLT group consisted of patients transfused with aplts, pooled plts, and RBCs, and the RBC group consisted of patients transfused with RBCs only (Table 3). The rate of TA-MC in this study was 50% in the FrWB group (3 of 6), 50% in the aPLT group (4 of 8), and 38% in patients receiving only RBCs (3 of 8). The rate of TA-MC in patients receiving only RBCs was not significantly different compared with patients in the FrWB group ($p = 0.60$) or the aPLT group ($p = 0.38$, Fig. 2).

DISCUSSION

Hematopoietic chimerism refers to the persistence of allogenic donor blood cells in a recipient. This chimerism has been categorized as MC (variably defined as less than 1%–5% donor cells) or macrochimerism (when the donor cells comprise a larger percentage).[17] MC can occur naturally, such as in pregnancy,[18] or artificially after solid organ transplantation.[19–21] During pregnancy, bidirectional fetomaternal DNA trafficking occurs through the trophoblasts of the placenta, leading to a state of MC, a mechanism by which pregnancy may avoid immunologic rejection by the mother. The number of women who have MC of fetal origin and the number of adults with circulating maternal cells is not cur-

Table 2 Microchimerism Demographics

Variable	No Microchimerism	Microchimerism	*P*
n (%)	16 (61.5)	10 (38.5)	—
Age (yr)	24 ± 7	24 ± 5	0.49*
Male (%)	16 (100%)	10 (100%)	1.0†
Injury Severity Score	19 ± 14	13 ± 7	0.11*
Hospital length of stay (d)	49 ± 41	41 ± 21	0.28*
Transfusion (%)	12 (75%)	10 (100%)	0.14†
Units of blood transfused	26 ± 26	30 ± 53	0.81*
Time from injury to blood draw (d)	42 ± 34	35 ± 22	0.29*
Time from last transfusion to blood draw (d)	24 ± 11	21 ± 12	0.27*

Age, Injury Severity Score, hospital length of stay, units of blood transfused, time from injury to blood draw, and time from last transfusion to blood draw data presented as mean ± SD.

* Student *t* test.

† Fisher exact test.

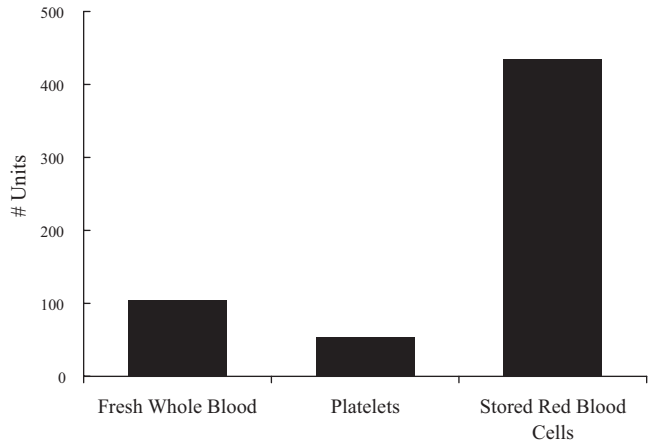

Fig. 1. *Transfusion products. The most common blood product transfusion was RBCs (455 units) followed by FrWB (105 units) and plts (54 units).*

rently known, but the long-term consequences of MC may place these patients at higher risk for the development of autoimmune disease.[22,23] An equally important phenomenon is the reciprocal migration of donor and recipient leukocytes after whole organ allografting. Transplantation causes the bone marrow-derived donor leukocytes normally found in the interstitial tissues of whole organs to migrate into the recipient circulation. In addition, a bidirectional exchange of recipient white blood cells can repopulate the interstitium of the transplanted solid organ, an event seminal to the acceptance of allografts and to the induction of donor-specific tolerance. The persistence of MC suggests that hematolymphopoietic precursor and stem cells are part of the "passenger" leukocyte population of organ grafts.[24]

Another mechanism resulting in the induction of MC is the use of blood transfusions. Several studies in different patient populations have documented the presence of TA-MC. In 1977, Schechter et al. documented the presence of circulating donor lymphocytes in six patients who received blood transfusions and demonstrated the persistence of donor lymphocytes up to 1 week after transfusion.[7] In addition, Lee et al. studied women who received blood transfusions from male donors and documented the presence of male donor leukocytes in these women between 4 and 7 days after transfusion.[25] Although the cells were cleared rapidly, the data represented the first independent corroboration of Schechter's work and suggested that "passenger lymphocytes" played a more active role in the mediation of transfusion effects than had been widely appreciated. In a more recent study, Kruskall et al. detected MC after allogenic RBC transfusions in 11% of HIV patients, though no MC was detected in these patients by 4 weeks after transfusion.[26]

TA-MC has also been identified in critically injured patients. Evidence of MC was documented in 10 female trauma patients who received blood from male donors. Up to 5% of all leukocytes in these patients were thought to have arrived from the male donors, and these cells were observed to persist for at least 1 year in two of the female recipients.[27] In addition, Utter et al. prospectively studied 45 trauma patients who underwent transfusions and sampled blood before hospital discharge to determine evidence of MC. They found that transfusion after trauma was associated with 53% of recipients having evidence of MC and that the transfusion was the source of this MC.[16] Our current study is in agreement with these reports and documents that transfused combat casualties had a 45% rate of TA-MC.

Leukoreduction of blood transfusions typically reduces the concentration of donor white blood cells from approximately 10^9 to approximately 10^6 white blood cells per liter. The universal application of leukoreduction for blood transfusions has resulted in a significant decrease in posttransfusion purpura and TA graft-versus-host disease (GVHD) in the United Kingdom.[28] However, leukodepletion of blood products has not affected the incidence of TA-MC in trauma patients. A recent study by Utter et al. documented that leukoreduction failed to prevent or even substantially reduce the likelihood of developing TA-MC. Patients transfused only with leukoreduced blood products had a 37% rate of TA-MC compared with a 28% rate of TA-MC in patients receiving nonleukoreduced blood products ($p = 0.43$).[29]

Blood transfusions are a well-known risk factor for increased morbidity and mortality in trauma.[30–32] One potential mechanism for this increased morbidity from blood transfusion is related to the storage time of blood. Zallen et al. identified mean age of blood, number of units older than 14 days, and number of units older than 21 days as independent

Table 3 **Blood Product Distribution and Microchimerism**

Blood Product	Microchimerism	No Microchimerism	Total
Fresh whole blood group			
Fresh whole blood (units)	25 (24%)	80 (76%)	105
Pooled platelets (units)	1 (7%)	13 (93%)	14
Stored red blood cells (units)	41 (19%)	176 (81%)	217
Apheresis platelet group			
Apheresis platelets (units)	12 (46%)	14 (54%)	26
Pooled platelets (units)	12 (86%)	2 (14%)	14
Stored red blood cells (units)	65 (45%)	80 (55%)	145
Stored red blood cell group			
Stored red blood cells (units)	45(48%)	48 (52%)	93

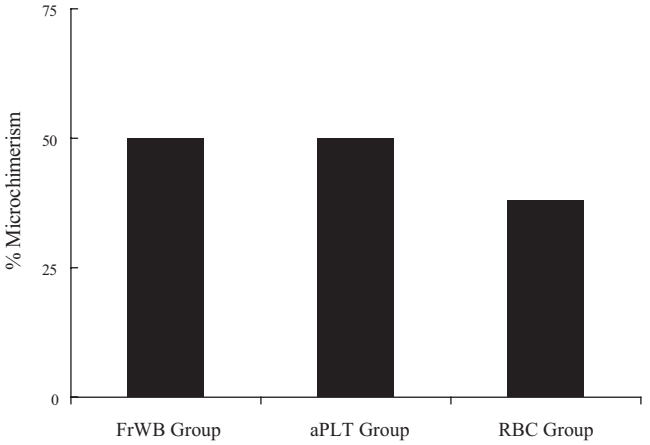

Fig. 2. *Blood products and prevalence of TA-MC. There was no significant difference in the prevalence of MC in patients receiving only RBC transfusions (RBC group) compared with patients receiving FrWB/pooled plts/RBC transfusions (FrWB group) or aplt/pooled plts/RBC transfusions (aPLT group). Fisher's exact test,* $p = 0.61$.

risk factors in the development of multiorgan failure on multivariate analysis.[33] Similarly, Offner et al. documented age of blood as an independent risk factor for major infections on multivariate analysis.[34] Age of blood also seems to be a factor in the development of TA-MC. In a recent study, patients who developed MC received blood units that had been stored for a significantly shorter time than those who did not develop MC, whether the storage time was characterized as the mean time of all units transfused or the minimum time determined by the most recently donated unit transfused.[16]

Another potential mechanism of this increased morbidity from blood transfusion is the presence of donor leukocytes. Their presence may result in complications, including fever-chill reactions, HLA alloimmunization, and mortality from GVHD.[35] The host defense of immunologically mediated and damaging set of reactions by genetically dissimilar cells is a phenomenon that was first described in the 1950s. Mice were given allogenic spleen cells after irradiation and they developed what is now known as GVHD as a "secondary disease" to differentiate it from the primary disease of radiation sickness. The term graft-versus-host reaction was introduced a few years later to describe the immunologic damage caused by an introduction of immunologically competent cells into an immunocompromised host.[36] Scientists then proposed the three conditions required for the development of GVHD, which have been minimally revised as: (1) the graft must contain immunologically competent cells, (2) the recipient must have autoimmunity and have major histocompatibility differences from the donor, and (3) the recipient immune response must be incapable of rejecting donor cells.[37–39] These three conditions are present in patients receiving blood transfusions who go on to develop TA-MC. GVHD developing as a result of transfusion is termed transfusion-associated GVHD (TA-GVHD). This disease process was published by Hathaway et al., and is known to have a more fulminant and fatal course compared with GVHD in transplantation patients.[40,41] TA-GVHD is extremely rare and most commonly occurs in immunodeficient patients, although isolated reports of TA-GVHD in immunocompetent patients have been documented.[42–44]

In summary, these preliminary data do not demonstrate a significantly increased rate of TA-MC in recipients of FrWB or plts compared with RBCs. Larger studies are needed to determine whether there is an increased rate of TA-MC in combat casualties transfused with FrWB. In addition, the overall 45% rate of TA-MC in transfused soldiers from Operation Iraqi Freedom and Operation Enduring Freedom warrants further study to ascertain possible clinical consequences such as GVHD or autoimmune disease syndromes.

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DISCUSSION

Dr. Philip C. Spinella (Connecticut Children's Medical Center, Hartford, CT): This study is valuable in that it supports previous literature that has demonstrated a high risk of TA-MC in trauma patients that may be increased by the transfusion of fresh blood products.

From recent research of FWB and aplt use in combat settings, we know that those who receive these products typically receive an increased total amount of blood products compared with patients who only receive RBCs. This occurs because of increased severity of injury in patients transfused FWB and aplts. I would be interested in seeing the results of the amount (percentage) of each blood product transfused and the number of donor exposures in each of the groups being compared. Although previous literature in small sample sizes has not detected an association with the amount of RBCs transfused and TA-MC, it may be worth exploring in this data set because increased donor exposures may increase the risk of TA-MC. If the FWB/RBC or FWB/aPLT/RBC group had increased number of donor exposures compared with the RBC-only group, this is an alternative reason that TA-MC may have occurred in those groups.

The most important question that this study presents is what is the clinical significance of a high incidence of TA-MC in trauma patients? TA-GVHD occurs most commonly in immunodeficient patients. It does occur rarely in immunocompetent patients typically as a result of similar HLA antigens between donor and recipient. Even though the risk of TA-MC is high in trauma patients and can be elevated with fresh blood products, the risk of TA-GVHD in trauma patients is not as clear. The United Kingdom hemovigilance scheme serious hazards of transfusion registry reported between 1995 and 2005 one case of TA-GVHD attributed to the transfusion of fresh RBCs (<5 days old) in over 25,000 transfusions. Trauma patients have altered immune states, which might increase the risk of GVHD from TA-MC, although Utter et al. (Transfusion. 2006;46:1863–1869) indicated that the high incidence of TA-MC in trauma patients has not been associated with increased risk of infection, multiorgan failure, or symptoms associated with GVHD. The

clinical significance of TA-MC in patients receiving either FWB or aplts becomes even more important when balanced with the increased 30-day survival that both of these products have been associated with independently in a study of over 500 massive transfusion patients as reported at this conference (J.G. Perkins, ATACCC, 2007). Although a high incidence of TA-MC is important to recognize, especially because its long-term effects are not well known, when put into perspective of improved 30-day survival for recipients of fresh products (FWB or platelets) and the large amount of laboratory and clinical evidence that older RBCs have diminished function and are associated with worse outcomes, the risk-to-benefit ratio of fresh or older products seems to favor the use of fresh blood products (RBCs $<$14 days of storage). Future research goals must include methods to decrease the risk of TA-MC and its potential adverse effects while maintaining the advantage of transfusing fresher more functional blood products to critically ill patients.

I have several questions for Dr. Dunne. What was the amount (percentage) of each blood product and the number of donor exposures for each group? What was the mean or median storage age of RBCs in each group? Did the severity of injury (ISS) correlate with the amount or degree of MC? And have longitudinal studies been performed to determine whether the degree of TA-MC changes in patients over a long period of time? Most importantly, in light of increased amounts of FWB or aplts being independently associated with improved 30-day survival for combat casualties requiring massive transfusion, what would your recommendations be to deployed military physicians who are contemplating the use of FWB or aplts for a casualty with hemorrhagic shock? Also, if you had a choice with what we know about the decreased function and adverse effects of older RBCs, would you prefer fresher or older RBCs to transfuse to severely injured combat casualties in shock?

CDR James R. Dunne (National Naval Medical Center, Bethesda, MD): Thank you Dr. Spinella for your comments. I will attempt to answer your questions in order. Your first question dealt with the percentage of blood products and the number of donor exposures. We have in fact performed this analysis on all the groups compared and have found results similar to those of Utter and colleagues. There was no significant difference in total number of donor exposures in patients who developed microchimersim compared with those who did not.

In reference to your second question regarding the age of packed RBCs, unfortunately we have been unable to obtain complete data on age of blood secondary to the patients being transfused at multiple hospitals throughout the medical evacuation process as well as the volume of blood transfused. The limited data we do have from in theater would suggest that the age of blood is not a factor in the development of TA-MC. However, these data are preliminary and incomplete and I hesitate to draw any conclusions based on it.

Regarding ISS and TA-MC, we found no significant difference in injury severity between patients who developed TA-MC compared with those who did not. These results are similar to other studies that also have failed to show a difference related to injury severity. We did not specifically look at the severity or degree or TA-MC, only the presence or absence of it.

I am not aware of any studies dealing with the evaluation of the severity of TA-MC over time. However, Lee and colleagues have data showing how the concentration of MC cells actually increase substantially in about half of the patients studied whereas the MC cells in the other patients gradually decreased. Their group has also documented the presence of MC cells in approximately 10% of World War II, Korean, and Vietnam veterans who were transfused for their combat injuries compared with $<$1% of age- or gender-matched controls.

Regarding the use of fresh whole blood and platelets, I think the data you have referred to is quite compelling and I would encourage their use by deployed physicians especially when component therapy is not available.

Finally, in reference to your last question, let me first state if any patient who is in shock needs blood then I recommend they get blood regardless of its age. Having said that, though, I think there is an abundance of data indicating that old blood is less effective and is a risk factor for increased morbidity, mortality, and increased resource utilization. The data regarding MC and age of blood are still emerging and the long-term clinical effects are still unproven, though TA-GVHD remains a concern. Therefore, I would recommend giving fresh blood in the situation you describe.